Title: **The tumor necrosis factor superfamily members TWEAK, TNFSF15 and fibroblast growth factor inducible protein 14 are upregulated in proliferative diabetic retinopathy**

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Abstract

Purpose: Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) and TNF superfamily member 15 (TNFSF15), members of the TNF superfamily, play important roles in the modulation of inflammation and neovascularization. TWEAK activity is mediated via binding to fibroblast growth factor-inducible molecule 14 (Fn14). We investigated the expression of TWEAK, Fn14 and TNFSF15 and the correlation between TWEAK levels and the levels of the inflammatory biomarker soluble intercellular adhesion molecule-1 (sICAM-1) in proliferative diabetic retinopathy (PDR). In addition, we examined the expression of FN14 and TNFSF15 in retinas of diabetic rats.

Methods: Vitreous samples from 34 PDR and 23 nondiabetic patients were studied by enzyme-linked immunosorbent assay and Western blot analysis. Epiretinal membranes from 14 patients with PDR were studied by immunohistochemistry. The retinas of rats were examined by Western blot analysis.

Results: We identified a significant increase in the expression of TWEAK, Fn14, TNFSF15 and sICAM-1 in vitreous samples from PDR patients compared to controls. A significant positive correlation was found between levels of TWEAK and levels of sICAM-1 ($r=0.3$, $p=0.02$). In epiretinal membranes, TWEAK and TNFSF15 protein expression was confined to vascular endothelial cells, monocytes/macrophages and myofibroblasts. Significant positive correlations were observed between the number of blood vessels expressing CD34 and the number of blood vessels expressing TWEAK ($r=0.670$; $p=0.017$) and TNFSF15 ($r=0.784$; $p=0.001$). The expression level of TNFSF15 was upregulated in the retinas of diabetic rats, whereas Fn14 was not upregulated.

Conclusions: Our findings suggest that TNFSF15 and the TWEAK/Fn14 pathway are novel mediators involved in persistent inflammation and modulation of pathological neovascularization associated with PDR.

Key words: proliferative diabetic retinopathy; inflammation; angiogenesis; TNF superfamily; TWEAK; TNFSF15; Fn14

Introduction

Ischemia-induced angiogenesis and expansion of extracellular matrix in association with the outgrowth of fibrovascular membranes at the vitreoretinal interface is the pathological hallmark in proliferative diabetic retinopathy (PDR). Persistent inflammation and neovascularization are critical for PDR progression. Recently, it was demonstrated that the processes of inflammation and angiogenesis are closely interconnected [1, 2]. Overexpression of proinflammatory and proangiogenic growth factors and cytokines has been observed in PDR [3-5]. Angiogenesis, the sprouting of new blood vessels from preexisting blood vessels, is a multistep process that involves cell proliferation, migration, tube formation of endothelial cells, remodeling of extracellular matrix, and functional maturation of the newly assembled vessels [6]. In addition, vasculogenesis, the *de novo* formation of blood vessels from circulating bone marrow-derived endothelial precursor cells, can contribute to new vessel formation in PDR [7, 8]. A key player of both these processes is vascular endothelial growth factor (VEGF), also called vascular permeability factor [9-11]. Nevertheless, it is likely that other factors also function as regulators of neovascularization in PDR, and the identification and characterization of these factors may result in the development of additional therapeutic agents.

Several cytokines belonging to tumor necrosis factor (TNF) superfamily were recently identified to play critical roles in the modulation of inflammation and neovascularization (12-16), two tightly intertwining biological processes [1, 2]. Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) activity is mediated via binding to fibroblast growth factor-inducible molecule 14 (Fn14), a member of the TNF receptor superfamily. TWEAK binding to Fn14 activates the nuclear factor (NF)-κB signal transduction pathway in multiple cell types, which has a central function in the generation of an inflammatory response [12-14]. Vascular endothelial growth inhibitor (VEGI), also known as TNF-like ligand 1A (TL1A), is designated as TNF superfamily member 15 (TNFSF15) [15, 16]. TWEAK and TNFSF15 are synthesized as type II-transmembrane proteins that can be cleaved to generate soluble factors with biological activity [12-16].

Recent studies revealed that TWEAK, acting via the Fn14 cell-surface receptor, is a multifunctional proinflammatory/proangiogenic cytokine [12-14]. TWEAK can stimulate numerous cellular responses including proliferation, migration, proinflammatory molecule production and angiogenesis [12-14]. TWEAK has been reported to be able to induce the

expression of the proinflammatory molecules matrix metalloproteinase-9 (MMP-9), intercellular adhesion molecule-1 (ICAM-1), E-selectin, interleukin (IL)-6, IL-8 and monocyte chemoattractant protein-1 (MCP-1) in multiple cell types [17-21], mediators which are involved in the pathogenesis of PDR. Likewise, a growing body of evidence supports a role for the TWEAK/Fn14 pathway in the pathophysiology of autoimmune and inflammatory diseases [12-14]. There is considerable evidence demonstrating a proangiogenic role for TWEAK. Soluble TWEAK promotes endothelial cell proliferation and migration *in vitro* [20, 22, 23]. TWEAK also induces neovascularization *in vivo* in a rat cornea model, where its activity was comparable to that of VEGF [23], TWEAK also enhances tumor angiogenesis [24] and a link between the TWEAK/Fn14 pathway and fibrosis has been demonstrated in a number of disease model systems [14].

In contrast to TWEAK, TNFSF15 is an endogenous inhibitor of angiogenesis produced largely by vascular endothelial cells and is a specific inhibitor of endothelial cell proliferation. TNFSF15 is able to enforce growth arrest on quiescent endothelial cells and to induce apoptosis of proliferating endothelial cells [15, 16, 25]. In addition, TNFSF15 can participate in the modulation of postnatal vasculogenesis by inhibiting bone marrow-derived endothelial progenitor cell mobilization, incorporation into tumor vasculature and differentiation into endothelial cells [26, 27]. Exogenous administration of recombinant TNFSF15 exhibits a highly potent inhibitory effect on tumor angiogenesis and tumor growth in animal models [28]. In addition, TNFSF15 helps to modulate the immune system by stimulating T cell activation, T helper 1 cytokine production and dendritic cell maturation [15, 16]. Recent studies have clearly shown that TNFSF15 is upregulated and possibly implicated in the pathogenesis of several chronic inflammatory conditions including inflammatory bowel disease [29-32], rheumatoid arthritis [33-37], psoriatic skin lesions [38], and atherosclerosis [39].

The expression of the TWEAK/Fn14 pathway and TNFSF15 in PDR has not been reported so far. In view of the emerging evidence for TWEAK/Fn14 pathway and TNFSF15 in modulating neovascularization and inflammation, we investigated their expression in vitreous fluid and epiretinal membranes from patients with PDR and studied the correlation between the vitreous fluid levels of TWEAK and the inflammatory biomarker soluble intercellular adhesion molecule-1 (sICAM-1). In addition, we investigated their expression in the retinas of diabetic rats.

Materials and Methods

Vitreous samples and epiretinal membranes specimens

Undiluted vitreous fluid samples (0.3 – 0.6 ml) were obtained from 34 patients with PDR during pars plana vitrectomy. The indications for vitrectomy were tractional retinal detachment, and/or nonclearing vitreous hemorrhage. The diabetic patients were 22 males and 12 females whose ages ranged from 18 to 86 years with a mean of 57.5±14.4 years. Twenty patients had insulin-dependent diabetes mellitus, and 14 patients had noninsulin-dependent diabetes mellitus. The control group consisted of 23 patients who had undergone vitrectomy for the treatment of rhegmatogenous retinal detachment with no proliferative vitreoretinopathy. Controls were free from systemic disease and were 15 males and 8 females whose ages ranged from 24 to 65 years with a mean of 45±15.4 years. Vitreous samples were collected undiluted by manual suction into a syringe through the aspiration line of vitrectomy, before opening the infusion line. The samples were centrifuged (500 rpm for 10 min, 4°C) and the supernatants were aliquoted and frozen at -80°C until assay.

Epiretinal fibrovascular membranes were obtained from 14 patients with PDR during pars plana vitrectomy for the repair of tractional retinal detachment. The severity of retinal neovascular activity was graded clinically at the time of vitrectomy using previously published criteria [40]. Neovascularization was considered active if there were visible perfused new vessels on the retina or optic disc and present within tractional epiretinal membranes. Neovascularization was considered inactive (involuted) if only nonvascularized white fibrotic epiretinal membranes were present. Active PDR was present in 5 patients and inactive PDR was present in 9 patients. Membranes were fixed in 10% formalin solution and embedded in paraffin.

The study was conducted according to the tenets of the Declaration of Helsinki. All the patients were candidates for vitrectomy as a surgical procedure. All patients signed a preoperative informed written consent and approved the use of the excised epiretinal membranes and vitreous fluid for further analysis and clinical research. The study design and the protocol were approved by the Research Centre and Institutional Review Board of the College of Medicine, King Saud University.

Rat streptozotocin-induced diabetes model

All procedures with animals were performed in accordance with the ARVO statement for the use of animals in ophthalmic and vision research and were approved by the Institutional Animal Care and Use Committee of the College of Pharmacy, King Saud University. Adult male Sprague-Dawley rats, 8-9 weeks of age weighting in the range of 250-300 g were overnight fasted and streptozotocin (STZ) (65 mg/kg in 50 mM sodium citrate buffer, pH 4.5; Sigma, St.Louis, MO) was injected intraperitoneally. Equal volumes of citrate buffer were injected in control (non-diabetic) animals. Measurement of blood glucose concentrations and body weight were started 3 days after injection of STZ. Diabetes was confirmed by assaying the glucose concentration in blood taken from the tail vein. Rats with glucose levels >250 mg/dl were categorized as diabetic. After 4 weeks of diabetes, animals were anesthetized by intraperitoneal injection of an overdose of chloral hydrate and sacrificed by decapitation. Retinas were dissected, flash frozen and stored at -70^0C until use. Similarly, retinas were obtained from age-matched nondiabetic control rats.

Enzyme-linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) kits for human TWEAK/TNFSF12 (Human TNF-related weak inducer of apoptosis, Cat No: DY1090) and human sICAM-1/CD54 (Human soluble Intercellular Adhesion Molecule-1, Cat No: DCD540) were purchased from R&D Systems, Minneapolis, MN. The minimum detection limit for sICAM ELISA kit was 0.096 nanograms/mL (ng/mL). The ELISA plate readings were done using Stat Fax 4200 microplate reader from Awareness Technology, Inc. Palm City, USA.

Measurement of TWEAK and sICAM-1

The quantification of human TWEAK and sICAM-1 in the vitreous fluid was determined using ELISA kits according to the manufacturer's instruction. For each ELISA kit, the undiluted standard served as the highest standard and calibrator diluents served as the zero standard. Depending on the detection range for each ELISA kit the supernatant vitreous fluids were adequately diluted with calibrator diluents supplied with ELISA kit.

For the measurement of TWEAK and sICAM-1, 100 µL of 4-fold diluted vitreous samples were analyzed in the respective ELISA assays. As instructed in the kit manual,

samples were incubated into each well of ELISA plates. Antibodies against TWEAK and sICAM-1 conjugated to horseradish peroxidase were added to each well of the ELISA plate. After incubation, substrate mix solution was added for colour development. The reaction was stopped by the addition of 2N sulfuric acid (R & D systems) and optical density was read at 450 nm in a microplate reader. Each assay was performed in duplicate. Using the 4-parameter fit logistic (4-PL) curve equation, the actual concentration for each sample was calculated. For the diluted vitreous fluids, the correction read from the standard curve obtained using 4-PL was multiplied by the dilution factor to calculate the actual reading for each sample.

Western blot analysis

To determine the TNFSF15 and Fn14 protein levels in the retinas of 10 non-diabetic and 10 diabetic rats, retinal tissues were homogenized in a Western lysis buffer (30 mM Tris-HCL; pH 7.5, 5mM EDTA, 1% Triton X-100, 250 mM sucrose, 1 mM Sodium Vanadate and protease inhibitor cocktail). The protease inhibitor used was "Complete without EDTA" (Roche, Mannheim, Germany). The lysate was centrifuged at 14,000X g for 15 min (4°C) and supernatants decanted and the protein concentrations were estimated using the Bio-Rad protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA). Protein samples were boiled in Laemmli's sample buffer for 10 min and equivalent amounts of protein (40-50 μg) were separated on 10-15% SDS-polyacrylamide gels (SDS-PAGE) and transferred onto nitrocellulose membranes. To determine the expression levels of TNFSF15 and Fn14 in the vitreous samples, equal volumes of vitreous samples were boiled in Laemmli's sample buffer (1:1, v/v) and separated on 10-15% SDS-PAGE and transferred onto nitrocellulose membranes. After protein transfer, the membrane was blocked (1.5 h, room temperature) with 5% non-fat milk and then incubated overnight at 4°C with rat monoclonal anti-TNFSF15 (1:300; Cat No: MAB-7441 R&D Systems, Minneapolis, MN) and goat polyclonal anti-Fn14 (1:300; Cat No: AF-1610, R&D Systems), After incubation with primary antibody, the membranes were washed and incubated at room temperature for 1.5 h with their respective secondary horseradish peroxidase-conjugated antibody. Membranes were again washed four times and the immunoreactivity of bands was visualized on a high-performance chemiluminescence machine (G: Box Chemi-XX8 from Syngene, Synoptic Ltd. Cambridge, UK) by using enhanced chemiluminescence plus Luminol (Sc-2048, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and quantified by densitometric analysis using image processing and analysis in GeneTools (Syngene by Synoptic Ltd.). To control for sample

loading and processing, the blots were stripped and incubated with a mouse monoclonal anti-β-actin antibody (1:2000, SC-2048, Santa Cruz Biotechnology, Inc.).

Immunohistochemical staining

For CD34, α-smooth muscle actin and TNFSF15 detection, antigen retrieval was performed by boiling the sections in citrate based buffer [pH 5.9 – 6.1] [BOND Epitope Retrieval Solution 1; Leica] for 20 minutes. For TWEAK detection, antigen retrieval was performed by boiling the sections in Tris/EDTA buffer [pH 9] [BOND Epitope Retrieval Solution 2; Leica] for 20 minutes. Subsequently, the sections were incubated with the monoclonal and polyclonal antibodies listed in Table 1. Optimal working concentration and incubation times for the antibodies were determined earlier in pilot experiments. The sections were then incubated for 20 minutes with a post primary IgG linker followed by an alkaline phosphatase conjugated polymer. The reaction product was visualized by incubation for 15 minutes with the Fast Red chromogen, resulting in bright-red immunoreactive sites. The slides were then faintly counterstained with Mayer's hematoxylin [BOND Polymer Refine Red Detection Kit; Leica].

Omission or substitution of the primary antibody with an irrelevant antibody from the same species and staining with chromogen alone were used as negative controls. Sections from patients with glioblastoma were used as positive controls for the immunohistochemical staining methods.

Quantitation

Immunoreactive blood vessels and cells were counted in five representative fields, using an eyepiece calibrated grid in combination with the 40x objective. These representative fields were selected based on the presence of immunoreactive blood vessels and cells. With this magnification and calibration, immunoreactive blood vessels and cells present in an area of 0.33 x 0.22 mm^2 were counted.

Statistical analysis

Data are presented as the mean ± standard deviation .The Mann-Whitney test and Student's t-test were use to compare means from two independent groups. Spearman's correlation coefficients were computed to investigate correlation between variables. A p-

value less than 0.05 indicated statistical significance. SPSS version 19.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

Results

Levels of TNFSF15, TWEAK, Fn14 and sICAM-1 in vitreous samples

With the use of ELISA, we demonstrated that TWEAK and sICAM-1were detected in all vitreous samples from patients with PDR (n=34) and control patients without diabetes (n=23). The mean levels of TWEAK in vitreous samples from PDR patients (3.4±1.9 ng/ml) were significantly higher than those in nondiabetic patients (1.4±1.06 ng/ml) ($p<0.0001$; Student's t-test). Similarly, the mean levels of sICAM-1 in vitreous samples from PDR patients (12.4±8.8 ng/ml) were significantly higher than those in nondiabetic patients (6.4±3.4 ng/ml). ($p<0.0001$; Mann-Whitney test). In the whole study group, there was a significant positive correlation between vitreous fluid levels of TWEAK and sICAM-1 ($r=0.3$, $p=0.02$).

With the use of Western blot analysis, we demonstrated that TNFSF15 and Fn14 were detected in all vitreous samples from patients with PDR and control patients without diabetes. Densitometric analysis of the bands demonstrated a significant increase in TNFSF15 ($p=0.01$; Mann-Whitney test) and Fn14 ($p=0.006$; Mann-Whitney test) expression in vitreous samples from PDR patients compared to control patients (Fig. 1).

Immunohistochemical analysis

To identify the cell source of vitreous fluid TNFSF15 and TWEAK, epiretinal membranes from patients with PDR were studied using immunohistochemistry. No staining was observed in the negative control slides (Fig. 2A). All membranes showed blood vessels positive for the panendothelial cell marker CD34 (Fig. 2B), with a mean number of 43.3±45.4 (range, 1-125). Intense staining for TNFSF15 was identified in all membranes and was noted in the cytoplasm of vascular endothelial cells and stromal cells (Fig. 2D, E). The number of immunoreactive blood vessels per surface of 0.33 x 0.22 mm^2 ranged from 2 to 55, with a mean number of 17.1±15.5. The number of immunoreactive stromal cells ranged from 6 to 110, with a mean number of 42.5±38.1. Intense cytoplasmic immunoreactivity for TWEAK

was present in vascular endothelial cells and stromal cells in all membranes (Fig. 2F, G). The number of blood vessels immunoreactive for TWEAK per 0.33 x 0.22 mm^2 ranged from 3 to 70, with a mean number of 21.7±20.7. The number of stromal cells immunoreactive for TWEAK ranged from 12 to 90, with a mean number of 46.8±25.0. The majority of TNFSF15- and TWEAK-positive stromal cells were monoctyes/macrophages and spindle-shaped cells (Fig. 2E, G). In serial sections, the distribution and morphology of spindle-shaped stromal cells expressing TNFSF15 and TWEAK were similar to those of spindle-shaped stromal cells expressing the myofibroblast marker α-smooth muscle actin (Fig. 2C).

The mean numbers of blood vessels expressing CD34, were significantly higher in membranes from patients with active PDR (98.4±25.6) than in membranes from patients with inactive PDR (12.8±8.4) (p=0.003; Mann-Whitney test). The mean numbers of blood vessels and stromal cells expressing TNFSF15 were significantly higher in membranes from patients with active PDR (33.0±13.5; 71.0±39.3, respectively) than in membranes from patients with inactive PDR (8.2±7.4; 26.7±28.3, respectively) (p=0.004; p=0.019, respectively; Mann-Whitney test) . The mean numbers of blood vessels expressing TWEAK, were significantly higher in membranes from patients with active PDR (41.3±23.6) than in membranes from patients with inactive PDR (11.9±10.2) (p=0.026; Mann-Whitney test). The difference between the mean numbers of stromal cells expressing TWEAK in membranes from patients with active PDR (62.5±18.9) and in membranes from patients with inactive PDR (38.9±24.9) was not significant (p=0.201; Mann-Whitney test).

The level of vascularization and proliferative activity in epiretinal membranes were determined by immunodetection of the panendothelial marker CD34. A significant positive correlation was detected between the number of blood vessels expressing CD34 and the numbers of blood vessels expressing TNFSF15 (r=0.784, p=0.001) and TWEAK (r=0.670, p=0.017). On the other hand, the correlations between the numbers of blood vessels expressing CD34 and the numbers of stromal cells expressing TNFSF15 (r=0.475, p=0.086) and TWEAK (r=0.458, p=0.134) were not significant.

Severity of hypercglycemia and effect of diabetes on retinal expression of TNFSF15 and Fn14 in experimental rats

After induction of diabetes with a single high doses of streptozotocin, the body weights of the diabetic rats were significantly lower and their blood glucose value were more

than four-fold higher compared with age-matched normal control rats (170±22 vs 270±28g and 453±32 vs 111±12 mg/dl, respectively).

The previous clinical findings were corroborated in the preclinical diabetes rat model. We quantified the expression of TNFSF15 and Fn14 in rat retinas by Western blot analysis. Densitometric analysis of the bands revealed a significant increase in TNFSF15 ($p=0.007$; Mann-Whitmey test) in diabetic retinas (n=16) compared to nondiabetic controls (n=16). However, the expression of Fn14 did not differ significantly between diabetic and nondiabetic controls (Fig. 3).

Discussion

In the present study, we report for the first time increased levels of TNFSF15, TWEAK and Fn14 in the vitreous fluid from patients with PDR as compared to nondiabetic control patients. These findings are supported by positive immunostaining for TNFSF15 and TWEAK in epiretinal membranes from patients with PDR. Furthermore, we demonstrated upregulated expression of TNFSF15 in the retinas of diabetic rats.

Although the functional significance of TNFSF15 in PDR remains to be clarified, one possibility is that TNFSF15 contributes to sustain the inflammatory process. For example, treatment with recombinant TNFSF15 caused induction of MMP-9, IL-8 and TNF-α in several cell types [29, 35, 39]. These mediators are implicated in the progression of PDR. Recent studies have clearly shown that TNFSF15 is upregulated and possibly implicated in the pathogenesis of several chronic inflammatory conditions including inflammatory bowel disease [29-32], rheumatoid arthritis [33-37], psoriatic skin lesions (38), and atherosclerosis [39]. The serum and synovial fluid levels of TNFSF15 are elevated in patients with rheumatoid arthritis [33-35] and TNFSF15 aggravated collagen-induced arthritis in mice [35]. Treatment with anti-TNF-α agents significantly decreased TNFSF15 serum levels [34]. Similarly, expression of TNFSF15 is increased in the mucosa of inflammatory bowel disease patients [29, 30, 32]. Several studies suggested that TNFSF15 is a severity factor and that upregulation of TNFSF15 expression can promote mucosal inflammation and gut fibrosis that is caused by increased collagen deposition and number of fibroblasts [31, 41]. In addition, a significantly higher rate of intestinal strictures was found in Crohn's disease patients with

higher TNFSF15 levels [31]. These findings suggest that TNFSF15 may be a pro-fibrogenic factor in addition to its role in inflammation.

Initially, TNFSF15 was thought to be produced only by endothelial cells, and its production was induced by the proinflammatory cytokines TNF-α and IL-1β [15, 16, 25]. Further studies have shown that it is also secreted by monocyte/macrophages, dendritic cells, $CD4^+$ and $CD8^+$ T cells, and synovial fibroblasts [30, 36-39]. Using immunohistochemistry, we demonstrated for the first time that TNFSF15 protein was specifically localized in vascular endothelial cells, mononuclear cells and myofibroblasts in epiretinal membranes from patients with PDR. In addition, TNFSF15 expression in PDR epiretinal membranes correlated with the activity of angiogenesis. Our results in PDR are in agreement with previous studies that demonstrated TNFSF15 expression by synovial fibroblasts and mononuclear phagocytes in synovial tissue of rheumatoid arthritis patients [36, 37], by endothelial cells and macrophage/foam cells in atherosclerotic plaques [39], by mononuclear and neutrophil infiltration, perivascular spindle-like cells, and endothelial cells in psoriatic skin lesions [38], and by macrophages and $CD4^+$ and $CD8^+$ lymphocytes in intestinal tissue specimens from patients with Crohn's disease [30].

It has also been reported that recombinant TNFSF15 is a potent inhibitor to suppress endothelial cell proliferation, angiogenesis and tumor growth [15, 16, 25, 28]. The effect of TNFSF15 activity on endothelial cells is cell-cycle dependent. TNFSF15 induces growth arrest in quiescent endothelial cells but induces apoptosis of proliferating endothelial cells [25]. Although endothelial cell proliferation is important for angiogenesis, induction of vascular cell apoptosis and involution are also necessary for vascular remodeling, and have been shown to precede tumor necrosis and neovascularization [42]. Our findings of expression of TNFSF15 by endothelial cells of proliferating microvessels in PDR epiretinal membranes suggest that TNFSF15 may have a role in angiogenesis control.

In the present study, we also showed that TWEAK and Fn14 expression was upregulated in the vitreous fluid from patients with PDR. Using immunohistochemistry, we demonstrated that TWEAK protein was specifically localized in vascular endothelial cells, mononuclear cells and myofibroblasts in PDR epiretinal membranes and that there was a significant correlation between the level of vascularization in PDR epiretinal membranes and the number of blood vessels expressing TWEAK. Similarly, a previous study reported TWEAK expression by synovial fibroblasts from patients with rheumatoid arthritis and

psoriatic arthritis [43]. It is as yet unclear why the statistical differences, observed in the levels of Fn14 in patients, were not observed in the animal model. One possible explanation is the fact that the rat model represents short term effects of an acute diabetogenic event, whereas in the patients the disease probably evolved over much longer time intervals, eventually years.

Fn14 is normally expressed at relatively low levels in healthy tissues. Thus activation of the TWEAK/Fn14 pathway is highly controlled by the inducible expression of this receptor. Fn14 can be highly induced by growth factors, including VEGF and fibroblast growth factor-2 [22] and the cytokines TNF-α, and IL-1β [13, 14]. The TWEAK/Fn14 pathway promotes the immune response through its ability to induce cytokines, chemokines, adhesion molecules and MMP-9 [17-21]. In the current study, we found a significant correlation between the vitreous levels of TWEAK and that of the inflammatory biomarker sICAM-1. This finding is consistent with previous studies that reported that TWEAK activates multiple cell types to upregulate the expression of ICAM-1 [19-21]. Therefore, persistent TWEAK/Fn14 pathway activation could play a role in the inflammatory response in PDR. Recent reports using rodent models of human disease have indicated that TWEAK-dependent Fn14 signaling can contribute to the clinical severity of autoimmune and inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, and inflammatory bowel diseases [12]. In addition, there is considerable evidence demonstrating a proangiogenic [12-14] and fibrogenic [12] roles for TWEAK. These proinflammatory activities, along with its ability to promote angiogenesis [12-14] and fibrosis [12], suggest that TWEAK may play a role in the pathogenesis of PDR.

Recent studies have indicated that the TWEAK/Fn14 axis signaling within the brain contributes to both cell death and an increase in the permeability of the blood-brain barrier during cerebral ischemia. The expression of TWEAK and Fn14 increases in the ischemic brain of stroke patients [44] and in animal models of cerebral ischemia [45]. During cerebral ischemia, TWEAK/Fn14 interaction activates the transcription factor NF-κB pathway and induces the expression of MMP-9 and proinflammatory cytokines and chemokines, recruitment of neutrophils, disruption of the blood-brain barrier and neurodegeneration [17, 18, 45, 46]. In analogy with all mentioned scientific information, our findings suggest that TWEAK/Fn14 upregulation in PDR may contribute to PDR progression by multiple

mechanisms: (1) induction of proinflammatory mediators; (2) breakdown of blood-retinal barrier; (3) promotion of angiogenesis and (4) induction of neurodegeneration.

In conclusion, we have provided evidence for increased expression of the TWEAK/Fn14 pathway and TNFSF15 in PDR. Our findings suggest that TWEAK/Fn14 and TNFSF15 are novel mediators involved in persistent inflammation and modulation of pathological neovascularization associated with PDR. Further studies will elucidate the relative importance of the TWEAK/Fn14 pathway and TNFSF15 *versus* other mediators in PDR pathogenesis and progression. Therefore, the present data add critical insights to explore the potential of these pathways as therapeutic targets.

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Conflict of Interest: The authors declare no conflict of interest.

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Table 1. Monoclonal and polyclonal antibodies used for immunohistochemical staining

Primary Antibody	Dilution	Incubation Time	Source*
• Anti-CD34 (Clone My10) (mc)	1/50	60 minutes	BD Biosciences
• Anti- α-Smooth muscle actin (Clone 1A4) (mc)	1/200	60 minutes	Dako
• Anti-TNFSF15 (Catalogue No. ab85566) (pc)	1/100	60 minutes	Abcam
• Anti-TWEAK (Catalogue No. ab37170) (pc)	1/100	60 minutes	Abcam

*Location of manufacturers: BD Bioscience , San Jose, CA, USA; Dako, Glostrup, Denmark; Abcam, Cambridge, UK

mc = monoclonal; pc = polyclonal

Legends to Figures

Figure 1. Comparisons of mean protein levels, deduced from band intensities, for tumor necrosis factor superfamily-15 (TNFSF15) and fibroblast growth factor-inducible molecule 14 (Fn14) in vitreous samples from patients with proliferative diabetic retinopathy (PDR) (n=16) and nondiabetic control patients (C) (n=15). A representative set of samples is shown. *The difference between the two means was statistically significant at the 5% level.

Figure 2. Proliferative diabetic retinopathy (PDR) epiretinal membranes immunostainings.

Negative control slide that was treated with an irrelevant antibody showing no labeling (A). Immunohistochemical staining for CD34 showing blood vessels positive for CD34 (B). Immunohistochemical staining for α-smooth muscle actin showing cytoplasmic immunoreactivity in spindle-shaped myofibroblasts (C). Immunohistochemical staining for tumor necrosis factor superfamily-15 showing cytoplasmic immunoreactivity in vascular endothelial cells (arrows), stromal cells (arrowheads) (D) and spindle-shaped cells (arrowheads) (E). Immunohistochemical staining for tumor necrosis factor-like weak inducer of apoptosis (TWEAK) showing cytoplasmic immunoreactivity in vascular endothelial cells (arrows), stromal cells (arrowheads) (F) and spindle-shaped cells (arrowheads) (G) (original magnification X40).

Figure 3. Comparisons of mean band intensity ratios for tumor necrosis factor superfamiy-15 (TNFSF15) and fibroblast growth factor-inducible molecule 14 (Fn14) in the retinas of diabetic (D) and control (C) rats. Each Western blot experiment was repeated at least 3 times with fresh samples.

*The difference between the two means was statistically significant at the 5% level.

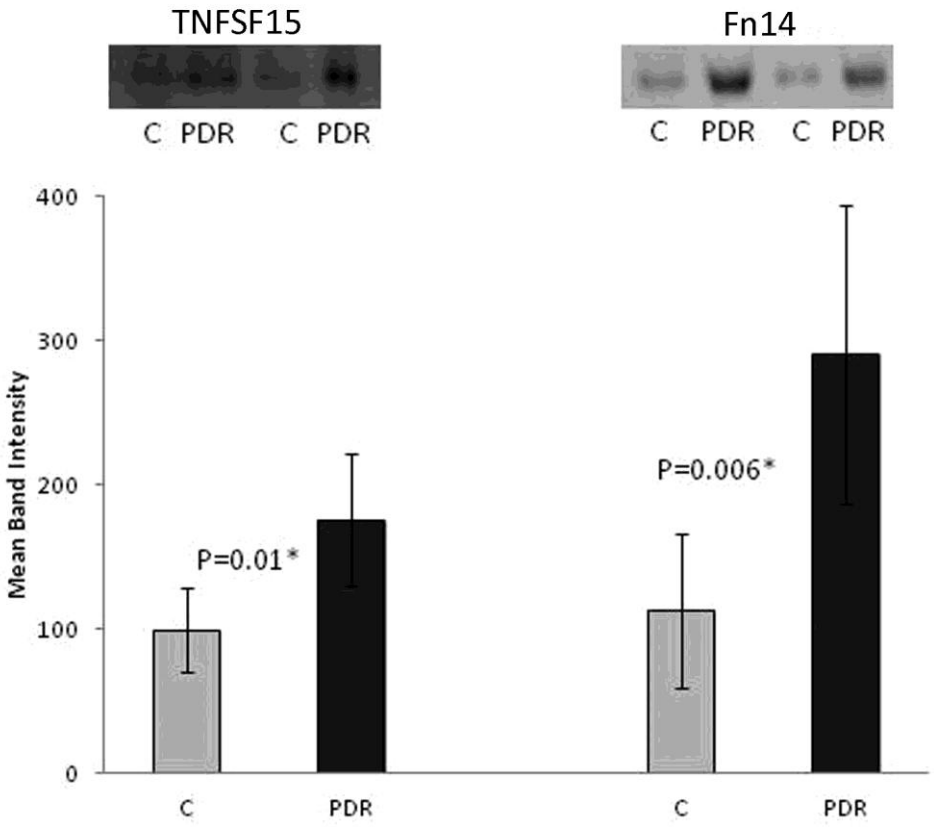
TNFSF15
Fn14
C PDR C PDR
C PDR C PDR
Mean Band Intensity
400
300
200
100
0
P=0.01*
P=0.006*
C
PDR
C
PDR

Figure 1

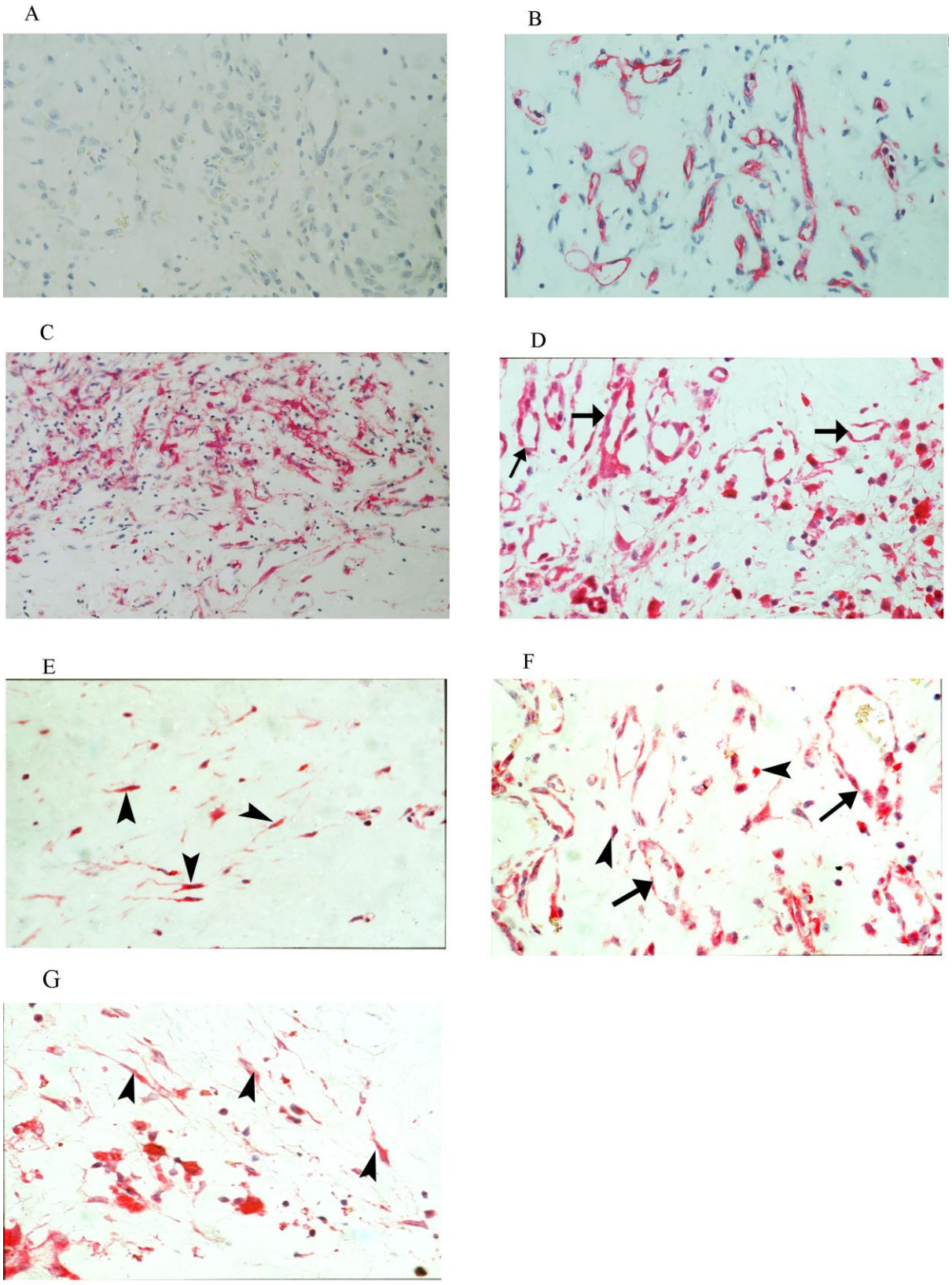

Figure 2

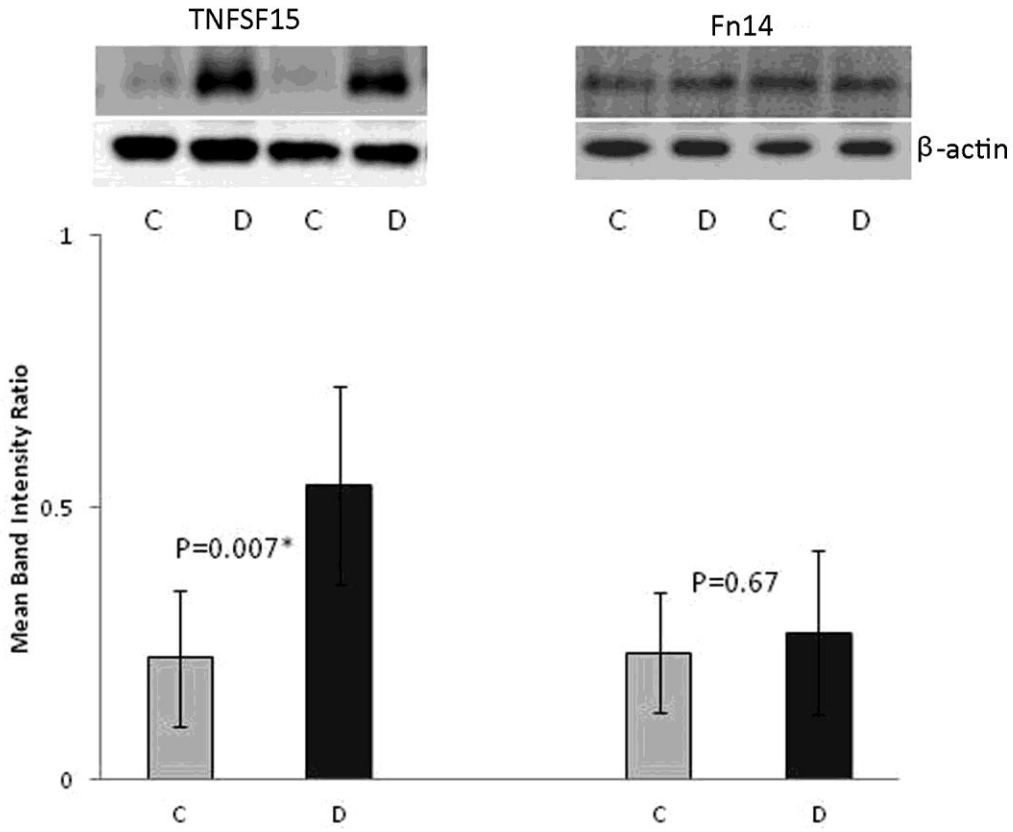

Figure 3

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